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(54) Title: METHOD FOR IDENTIFYING AN UNKNOWN ALLELE**(57) Abstract**

The invention provides a method for identifying an unknown allele of a polyallelic gene, which method comprises (i) contacting the unknown allele with a panel of probes, each of which recognises a sequence motif that is present in some alleles of the polyallelic gene but not in others; (ii) observing which probes recognise the unknown allele so as to obtain a fingerprint of the unknown allele; and (iii) comparing the fingerprint with fingerprints of known alleles. The use of a panel of probes which each recognises a different motif allows identification of which motifs are present in the unknown allele. The alleles of the polyallelic gene each have a unique combination of motifs and so identification of this combination (or "fingerprint") leads to identification of the unknown allele.

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METHOD FOR IDENTIFYING AN UNKNOWN ALLELE

The invention relates to a method and a kit for identifying an unknown allele of a polyallelic gene.

5

1. Background to the invention

1.1 General Introduction

10 Many genes exist as multiple alleles which differ from each other by small differences in sequence. It is sometimes desirable to identify an unknown allele of a polyallelic gene. For example, such identification is often necessary to match the alleles of the human leucocyte antigen (HLA) genes in a prospective donor and a prospective recipient in
15 a tissue or organ transplant operation; if the donor and recipient have the same HLA alleles, the probability of the recipient rejecting the donor's tissue is greatly reduced.

20 However, it can be a difficult task to identify precisely an unknown allele of a polyallelic gene because two alleles can differ from each other by as little as one nucleotide. The difficulties are increased in genes which have a very large number of different alleles, such as the major
25 histocompatibility complex (MHC) genes (e.g. the HLA class I genes which have 222 known alleles).

Up to date the most favourable bone marrow transplant (BMT) and kidney transplant results have been obtained using
30 sibling donors who are genotypically HLA-identical to the recipient but such donors are available for only about 30% of patients⁽¹⁻⁵⁾. BMT using unrelated donors can be successful, but these transplants have higher rates of graft failure, increased incidence and severity of Graft
35 versus Host Disease and more frequent complications related to delayed or inadequate immune reconstitution⁽⁴⁾.

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New molecular biological methods for detection of genetic polymorphism currently provide an opportunity to improve matching of unrelated donors as well as a research tool to investigate the relationship between genetic disparity and transplant complications. These molecular typing methods include sequence-specific amplification, hybridisation with oligonucleotide probes, heteroduplex analysis, single strand conformation polymorphism analysis and direct nucleotide sequencing.

Each of these molecular approaches has been used for routine HLA class II typing ⁽⁶⁾, but a variety of reasons related to the HLA class I gene structure has complicated and made relatively unsuccessful their application to class I typing. The reasons for these complications are the extensive polymorphism of class I and the degree of sequence homology between the A, B and C loci of class I. In addition, sequence homology between class I classical and non-classical genes and the reported 12 pseudo genes can cause problems for specific locus amplification ⁽⁷⁾.

The low occurrence of "allele specific" sequences at polymorphic sites is a feature of the HLA class I genes that has limited the resolution of all current DNA typing approaches. An "allele specific" sequence is a sequence that is only present in one allele and can therefore be used to distinguish the allele from other alleles. The occurrence on more than one exon of the specific sites for determining the allelic specificity causes additional problems in the identification of individual alleles. As a result, there is at present no single method of typing which can identify all HLA class I alleles of high resolution, see Table A below.

Comparison of some of the currently used DNA typing methods for HLA class I alleles

Table A

Method	Reference	HLA locus	Reagents	Resolution
SSO	Osato et al '96 Tissue Antigens 47:93-101	A	91 probes	High
SSO	Fernandez-Villa '95 Tissue Antigens 45:153-168	B	99	Medium-high
SSO	Levine/Yang '94 Tissue Antigens 44:174-183	Cw	64 probes	Medium-high
SSP	Bunce et al '95 Tissue Antigens 46:355-367	A,B,Cw	104 primer mixes	Low (\geq serology)
SSP	Bunce/Weish '94 Tissue Antigens 43:7-17	Cw	22 primer mixes	Low (=serology)
SSP	Krausa et al '93 Tissue Antigens 42:91-99	A9, 10, 19, 28	30 primer mixes	Medium
SSP (nested)	Krausa et al '95 Tissue Antigens 45:223-231	A2	15 primer mixes	High
PCR-RFLP	Tatari et al '95 Proc Natl Acad Sci 92:8803-7	Cw (partial, 23)	3 primer pairs, 11 endonucleases	High
SSP-SEQUENCING	Blasczyk et al '95 Tissue Antigens 46:86-95	A (40 alleles)	27 primer pairs automatic sequencing with fluorescent tracers	High
SSP-SEQUENCING	Petersdorf/Hansen '95 Tissue Antigens 46:73-85	B (98 alleles)	5 primer pairs automatic sequencing with fluorescent tracers	Medium-high
JRSTO	Arguello et al '96 Proc Natl Acad Sci 93:10961-5	A, B, Cw (201 alleles)	3 primer pairs 40 probes	High

Methods for allele separation

1.2 Sequence specific primer amplification (PCR-SSP)

5 This method utilises both the group-specific and, when present, allele-specific sequence sites in PCR primer design. The SSP design is based on the amplification refractory mutation system (ARMS), in which a mismatch at the 3' residue of the primer inhibits non-specific
10 amplification^(8,9).

Although each SSP reaction may not individually provide sufficient specificity to define an allele, the use of combinations of sequence specific primers allows the
15 amplification of their common sequences to give the desired specificity.

However, despite its high accuracy, PCR-SSP is only in some cases more informative than serology. The reason for this
20 is the low occurrence of allele specific sequence motifs in the exons and this limitation has stimulated a vast amount of research into the identification of allele specific motifs even in the intron sequences⁽¹⁰⁾. However, up to date this approach has not contributed considerably to the
25 identification of more alleles.

Another limitation of this method is that it detects a limited number of polymorphic sequences which are utilised to predict the entire sequence. If an unknown allele is
30 present in a particular sample this extrapolation may be incorrect.

In addition, the successful use of the technique relies on group specific amplification and therefore prior knowledge
35 of broad HLA specificity is needed.

1.3 Single strand conformation polymorphism (SSCP)

This technique is based on the electrophoretic mobility of single stranded nucleic acids in a non-denaturing polyacrylamide gel, which depends mainly on sequence-related conformation⁽¹¹⁻¹³⁾. The technique can be employed for isolating single alleles which could then be used for further manipulation and analysis such as direct sequencing. The pattern of bands obtained after electrophoresis may be diagnostic for an allele^(14,15).

The major disadvantage of SSCP is the tendency of DNA single strand to adopt many conformational forms under the same electrophoretic conditions resulting in the presence of several bands from the same product; this makes the identification more difficult. In addition there is a high degree of variation and inconsistency in the sensitivity of this method for detecting mutations or allelic variations and there is a physical limitation in the size of the DNA fragment which is of the order of 200-400 base pairs (16).

1.4 Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE) (17,18)

The underlying principle of both techniques is the difference in the degree of melting between two alleles (double stranded DNA) which results in a reduction of mobility of the DNA fragments in polyacrylamide gels containing a denaturing reagent (DGGE) or a temperature gradient (TGGE).

Both techniques have been used frequently for screening mutations in genetic systems with one or two variants. They are only rarely used for the separation of alleles in highly polymorphic systems such as HLA.

Both techniques require specific conditions for a particular system under investigation and, in addition,

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where two alleles share common sequence segments with low melting points they may not always be differentiated. The simultaneous melting of both alleles will produce very similar retardations.

5

1.5. Cloning of DNA

This is the classical method of preparation of a single sequence, i.e. the sequence derived from a single allele.

10 A variety of constructs has been used to introduce the required DNA fragment into a plasmid and grow sufficient copies for analysis. This method yields pure samples of the analyte, but is time consuming to perform and several clones are normally tested to ascertain the homogeneity of
15 the product.

Methods for the identification of alleles

1.6 Heteroduplex analysis

20

Fully matched DNA duplexes are more stable than those with base mismatches. Instability of the duplex increases with the number of nucleotide mismatches; these cause formation of loops and bends in the linear DNA fragment which produce
25 an increasing "drag effect" in polyacrylamide gels which retard the affected migrating bands⁽¹⁹⁻²¹⁾.

Mismatched DNA hybrids (heteroduplex) may be formed at the end of each PCR cycle between coamplified alleles from a particular locus or loci due to primer cross reaction at
30 sites with similar sequences. During the annealing stage of each cycle of the PCR, a proportion of sense strands of each allele may anneal to anti-sense strands of different alleles. The banding pattern obtained in PAGE analysis can
35 be useful for identifying the alleles involved in the reaction⁽²²⁻²⁴⁾.

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Heteroduplex analysis is an approach that has been utilised to compare HLA genes of a particular donor and recipient. HLA genes are amplified, denatured (melted into single strands) and mixed together under conditions that promote
5 renaturation to form double stranded molecules. If the HLA genes of a donor and recipient are similar but not identical, heteroduplexes will form consisting of one strand of an allele of donor origin and a second strand from a different allele of recipient origin^(25,26). The
10 sensitivity of this method can be increased by adding DNA from an HLA allele that is not present in the donor or recipient.

The major advantage of heteroduplex analysis is that it is
15 relatively easy and inexpensive. Limitations of this approach include inability to detect certain HLA disparities, potential detection of irrelevant silent mutations and lack of specific information regarding the nature of the alleles involved.

20

Up to date this approach has been used for HLA class II typing with limited success. Its application to class I typing has not been successful.

25 1.7 Sequence specific oligonucleotide probes (PCR-SSO)

SSO typing involves amplification of HLA alleles from a particular locus followed by hybridisation with a panel of oligonucleotide probes to detect polymorphic sequences that
30 distinguish one allele or group of alleles from all others. In polymorphic systems a one step operation may not always differentiate all the known alleles; selected primers can be used to achieve amplification of individual alleles which are then identified by specific probes. This second
35 stage of oligotyping is often referred to as high resolution oligotyping⁽⁶⁾.

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The advantages of the PCR-SSO method are specificity, sensitivity, simplicity, reproducibility, and it is relatively inexpensive to operate and allows simultaneous processing of many samples. This approach has been applied successfully, for example to typing of HLA class II alleles.

The major methodological drawback of this approach is that the complexity of the technique is directly related to the number of alleles under investigation and the presence of two alleles in the heterozygous condition can complicate the identification process.

Published oligotyping methods could result in incorrect interpretation of data if certain combinations of recently discovered alleles are present in a specimen. It is therefore necessary to update the reagents used in the identification step.

Several typing approaches for HLA-A and B based on PCR-SSO have been published; these typically require over 40 and 90 probes respectively ^(27,28). The operation of these methods is time consuming and the resolution obtained is only moderate.

1.8 Nucleotide Sequencing

DNA templates for sequencing can be produced by a variety of methods, the most popular being the sequencing of cloned genomic or cDNA fragments, or the direct sequencing of DNA fragments produced solely by PCR (as in 1.2 above). These templates represent a single sequence derived from one haplotype. Alleles from both haplotypes of a heterozygous sample may be co-amplified and sequenced together using locus-specific PCR primer.

The recent availability of computer software, which allows

the user to align the derived sequence against established sequence libraries, has facilitated the analysis and allele assignments for heterozygous samples in which both templates are sequenced at the same time⁽²⁷⁾. The effectiveness of this method depends on the amount and frequency of ambiguous heterozygous combinations; for example there are many HLA class II alleles that when present together in one sample cannot be differentiated by this method. The number of such ambiguous combinations of allele sequences is even greater for HLA class I alleles.

Up to date two HLA class I typing approaches based on direct sequencing have been published. Both require serology information followed by allele specific PCR amplification and then direct sequencing^(14,30). More recent practice, however, is to amplify DNA fragments without prior knowledge of the allele groups and to use locus specific PCR amplification. Theoretically these approaches should give the highest resolution, but they are beset by ambiguous sequence combinations which cannot be resolved satisfactorily and in practice these methods are expensive and difficult to perform routinely.

2. Analysis of the HLA class I polymorphism

Genetic recombination plays a key role in the generation of HLA alleles. This is supported by pairwise comparison of the nucleotide sequences. The most closely related pairs of alleles usually differ by localised clusters of substitutions for which both sequence motifs can be found in other alleles. This pattern implicates interallelic conversion or double recombination as the diversifying mechanism⁽⁷⁾. Although the vast majority of such events appear to involve recombination between alleles of the same locus, there are several cases that involve recombination between alleles of different loci⁽³¹⁾.

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In comparison to the many pairs of alleles that differ by localised clusters of substitutions, few pairs differ by point substitutions and of these only a handful differ by a substitution that has not been found in another allele.

5 Thus, it appears that the rate at which point mutations create new alleles is slower than the rate at which new mutations are subsequently recombined with existing mutations (Figure 1).

10 Comparison of allelic HLA class I sequences⁽³²⁾ reveals substitutions throughout the coding region. There is, however, a higher frequency of substitutions within exons 2 and 3 which encode the $\alpha 1$ and $\alpha 2$ domains of the HLA molecule. In comparing pairs of HLA-A, B and C alleles
15 only 2 pairs out of a total of 6,460 possible combinations can not be distinguished on the basis of nucleotide sequences in exons 2, 3 and 4. However, if the comparison is restricted to exons 2 and 3 this number only increases to 5 pairs of ambiguous sequences. By contrast, when
20 comparison is restricted to either exon 2 or exon 3 alone then the number of ambiguous pairs increases significantly (Table B). This observation is relevant to the design of DNA-based methods for class I typing because it shows that for practical purposes all alleles can be discriminated on
25 the basis of sequence analysis of exon 2 and 3. Although there is some polymorphism in exon 4 encoding the $\alpha 3$ domain, mostly in HLA-A alleles, incorporating the information from exon 4 into the above analysis does not significantly increase the number of pairs for which the
30 alleles can be discriminated.

TABLE B

Discriminating HLA-A, B, C alleles on the basis of partial sequences

Pairs without						
	Number of pairs	Exon 2, 3 or 4 differ.	Exon 2 or 3 differences	Exon 2 differ.	Exon 3 differ.	Exon 4 differ.
HLA-AXHLA-A	1081	0	1	26	28	129
HLA-BXHLA-B	4851	2	3	117	89	880
HLA-CXHLA-C	528	0	1	12	7	61

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In the development of PCR-based methodologies for the detection of alleles, one of the most important steps is the identification of primer sequences unique for the target gene which includes all polymorphic sites of interest in the amplified fragment, which should also be manageable in length. Typing of the polymorphic sites in exons 2 and 3 would facilitate the identification of all recognised alleles of HLA-A, B and C loci, with 5 exceptions, if suitable locus-specific amplification could be achieved.

Specificity of the primers should ensure the effective amplification of target gene fragments. In practice however, trace amplification of competing, cross-hybridising templates may also take place. In addition, due to the shared polymorphic sequence motifs between class I alleles of all three loci, non-specific coamplification of the DNA fragments would hinder specific identification. In practice, it would therefore be advantageous to use a method that allows the separation of the desired product from the undesirable PCR fragments.

Within exons 2 and 3 of the HLA-A, B and C genes there are only a few locus specific sites which are located primarily in the central region of each exon which would restrict the amplification to incomplete exon fragments. As discussed above, this would reduce the allele specific information necessary for the identification of all allelic variants.

The two polymorphic exons are flanked by introns 1 and 3, and separated by intron 2. Thus, the ideal location for primer sites to amplify exons 2 and 3 together as one fragment would be within introns 1 and 3.

Cereb and collaborators⁽³³⁾ have described primer sequences located in the first and third introns which can be used for locus-specific amplification of the entire exon 2 and 3

region of the HLA-A, B and C genes in one fragment. Their data indicated that the primers used in that study were effective in the amplification of HLA-A, B and C genes. Furthermore, the amplification was truly locus-specific, as
5 assessed by hybridisation with locus-specific, group-specific, and allele-specific oligonucleotide probes.

3. Summary of the Invention

10 The invention provides a method for identifying an unknown allele of a polyallelic gene, which method comprises

(i) contacting the unknown allele with a panel of probes, each of which recognises a sequence motif
15 that is present in some alleles of the polyallelic gene but not in others;

(ii) observing which probes recognise the unknown allele so as to obtain a fingerprint of the
20 unknown allele; and

(iii) comparing the fingerprint with fingerprints of known alleles.

25 The invention also provides a kit for identifying an unknown allele of a polyallelic gene, which kit comprises a panel of probes, each of which probes recognises a sequence motif that is present in some alleles of the polyallelic gene but not in others. (The same motifs may also occur in
30 other loci in linked gene complexes with similar exon/intron structures.) The kit preferably also comprises a database which indicates which probes in the panel recognise each allele of the polyallelic gene.

35 The use of a panel of probes which each recognises a different motif allows identification of which motifs are present in the unknown allele. The alleles of the

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polyallelic gene (and alleles of other genes in a linked complex) each have a unique combination of motifs and so identification of this combination (or "fingerprint") leads to identification of the unknown allele. Thus, the invention allows identification of alleles of polyallelic genes, such as the HLA class I genes, which may not contain "allele specific" sequences (i.e. individual sequences which are unique to one particular allele).

10 4. Brief Description of the Drawings

Figure 1 is a schematic representation of a polyallelic gene which has evolved by recombination events and to which the invention can be applied. See the Detailed Description of the Invention for more details.

Figure 2A shows a schematic overview of an embodiment of the "Complementary Strands Analysis" (CSA) technique that can be used to purify an allele for use in the method of the invention.

Figure 2B shows results of this CSA technique. In particular, Figure 2B shows an autoradiograph of the separation of HLA-A, B and Cw alleles from three International Histocompatibility Workshop cell lines by PAGE. Individual bands are eluted from the gel and used for subsequent analysis. Each band is a purified product from a single allele.

Figure 3 shows the hybridisation pattern of an URSTO probe (number 37 from Table 1) with HLA-A, B and C allele products. DNA from 15 IHW cell lines was processed by complementary strand analysis into allelic products. These were blotted on nylon membranes and was hybridised with URSTO probes (here 37). After washing the dots were developed and chemiluminescence were captured by autoradiography. In every case the presence of a signal

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corresponds with the hybridisation patterns described in Table 2. The unique aspect of this method is that the alleles of the three loci can be identified simultaneously by the analysis of the 40 membranes. 1-15 cell lines, C
5 control DNA product.

Figure 4 shows an HLA class I analysis of four cell lines with 12 URSTO probes.

10 HLA types of the cell lines:

- a. L0541265 A*0101, B*0801, Cw*0701;
- b. STIELIN A*0101, B*0801, Cw0701
- c. LBUF A*3001, B*1302, Cw0602
- d. BER A*0201, B*1302, Cw*0602

15

HLA-A, B and Cw alleles are blotted on the same membrane each membrane is hybridised with one probe. Locus specific amplification and allelic separation of the amplified fragments were performed as described in the Example below.

20 DNA was applied to nylon membranes and these were hybridised with URSTO probes, and after washing the chemiluminescence was recorded by autoradiography.

5. Detailed Description of the Invention

25

The invention can be applied to any polyallelic gene system in which there are motifs that are present in some alleles but not in others. The invention is mainly applicable to polyallelic systems that have evolved by recombination

30 events and/or by gene conversion in polygenic linked complexes. Examples of genes to which the invention can be applied are the mammalian MHC genes (e.g. the HLA class I and class II genes), the T cell receptor genes in mammals^(36,37), TAP, LMP, ras, nonclassical HLA class I

35 genes, human complement factor genes C4 and C2, Bf in the HLA complex, and genes located in mitochondrial DNA, bacterial chromosomes and viral DNA.

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Figure 1 illustrates a motif pattern that could have evolved from two alleles, each with four motifs (rectangles), of an ancestral gene. There are also allele specific sequences on each allele (diamond shape) which may have evolved from point mutations. The allele specific sequences are targets for SSO and SSP type allele identification techniques, but a minimum of 13 probes would be needed. In the method of the invention, only four probes (indicated on the top of the Figure) would be required to type the entire range. New coherent patterns would indicate new unknown alleles as shown at the foot of the Figure.

Identification of the alleles is by the presence or absence of hybridisation (+ or - respectively) of the probes as shown in Figure 3. Lack of binding, i.e. "-" in this system, is an important signal for pattern formation. No pattern is repeated in this 16 allele system and therefore each of the 16 alleles can be unambiguously identified by the invention.

The invention is particularly applicable to HLA class I genes. Comparison of HLA-A, B, C allelic sequences reveals a patchwork pattern in which an individual allele comprises a unique combination of sequence motifs, each of which is shared with other alleles, and only a few alleles have a specific sequence that is not present in other alleles (see Arnett and Parham (1995) *Tissue Antigens* 45 217-257). Many authors agree that this characteristic of the HLA class I genes has limited the resolution of all current DNA typing approaches. This feature itself has been exploited to facilitate the identification of all known class I alleles.

Comparison of the sequences of all known HLA class I alleles has led to the realisation that certain sequence motifs with one or more base substitutions recur in the same position in a locus and also in the same position in

another locus. Each allele contains a unique combination of these motifs; this feature is universal in all polyallelic genes that have evolved mainly through recombination events and/or by gene conversion. It is therefore possible to identify the alleles of such genes by a limited number of selected recurring motifs.

Analysis of these common motifs in the HLA class I complex on human chromosome 6 has led to the conclusion that by selecting a limited number of motifs it would be possible to identify all known alleles of this system by unique hybridisation patterns from this selected panel. Table 1 gives examples of sense probe sequences that identify these motifs. The probes could equally well have the antisense sequences.

In essence therefore this method differs from any other hitherto described method in that it does not target allele specific regions of the gene (cf SSO and SSP) but utilises recurring motifs which in specific combinations are unique for each allele.

A very large number of allele specific motif patterns can be generated with probes. The number of motif patterns generated by these oligonucleotides are sufficient to identify at least 201 HLA class I alleles. The sequences of 40 oligonucleotides are given in Table 1 and the expected patterns shown in Table 2. Table 3 shows the location and distribution of the 40 probes in HLA class I genes.

The selection of the target motifs for these probes ensures that for a coherent pattern no two probes for the same sequence location can hybridise with the product from a single allele. Incoherent patterns indicate an error in the amplification or separation stages.

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For unambiguous pattern identification it is usually necessary to analyse the alleles individually. The use of Complementary Strands Analysis (see below) is provided as a means of separating amplified alleles from each other.

Table 1

Nucleotide sequences and the sites of URSTO probes for the HLA class I genes

ID	Sequence	Location
2	GGG CCG GCC GCG GGG AGC	113-130
3	CTC ACA GAT TGA CCG AGT	282-299
4	CGG ATC GCG CTC CGC TAC	307-324
5	TAC CTG GAG GGC CTG TGC	547-564
6	CAG AGG ATG TAT GGC TGC	358-375
7	ACA CCC TCC AGA GGA TGT	350-367
8	CAG AGG ATG TTT GGC TGC	358-375
9	CGA CGT GGG GCC GGA CGG	375-392
10	CTC ACA TCA TCC AGA GGA	347-364
11	TGT ATG GCT GCG ACC TGG	365-382
12	CCA GCA GGA CGC TTA CGA	411-428
13	GTG CGT GGA CGG GCT CCG	561-578
14	GCG GAC ACG GCG GCT CAG	478-495
15	GGA GCA GTG GAG AGC CTA	531-548
16	GGA GCA GTT GAG AGC CTA	531-548
17	GTG CGT GGA GTG GCT CCG	561-578
18	GGA GCA GCT GAG AGC CTA	531-548
19	AGG GGC CCG AGT ATT GGG	236-253
20	GGC CCG ACG GGC GCC TCC	382-400
22	TCC CCG GGC ATA ACC AGT	401-418
23	ACC AGT TCG CCT ACG ACG	413-430
24	ATT GGG ACC GGA ACA CAC	248-265
25	TAC CTG GAG GGC ACG TGC	557-574
26	TGT ATG GCT GCG ACG TGG	365-382
28	GCC CAG TCA CAG ACT GAC	277-292
29	ACC GAG TGG ACC TGG GGA	293-310
30	CGG AAC CTG CGC GGC TAC	307-324
31	ATT TCT ACA CCT CCG TGT	92-109
32	GCC CGT GTG GCG GAG CAG	520-537
33	GAT CTC CAA GAC CAA CAC	267-284
34	TGA CCA GTC CGC CTA CGA	411-428
35	AAC ACA CAG ATC TAC AAG	259-276
36	CGC GGG CGC CGT GGG TGG	212-229
37	AGA TAC CTG GAG AAC GGG	580-597
40	TCT CAC ACC CTC CAG	346-360
41	ACC AAC ACA CAG ACT GAC C	277-295
42	GGC GCA GCA GCG GAG A	528-543
43	CAG GAC GCC TAC GAC GGC	415-432
44	GAG GAC CTG CGC TCC TGG	454-471
45	GAA GGA GAC GCT GCA GCG	597-614

ID, identification number.

TABLE 2

Allele specific hybridisation patterns

A*0101	2	9	13	19	26	37	42	43	45
A*0102	9	13	19	26	37	42	43	45	
A*0201	2	6	16	17	25	26	29	37	45
A*0202	2	6	7	15	17	25	26	29	37 40 45
A*0203	2	6	15	17	25	26	29	37	45
A*0204	2	16	17	25	26	29	37	45	
A*0205	2	6	7	15	17	25	26	29	31 37 40 45
A*0206	2	6	16	17	25	26	29	31	37 45
A*0207	2	16	17	25	29	37	45		
A*0210	2	8	16	17	25	29	31	37	45
A*0211	2	3	6	16	17	25	26	29	37 45
A*0212	2	6	17	25	26	29	37	45	
A*0213	2	6	17	25	26	29	37	45	
A*0214	2	6	7	16	17	25	26	29	31 37 40 45
A*0215N	2	16	17	25	29	37	45		
A*0216	2	6	16	17	26	29	37	45	
A*0217	2	16	17	25	29	37	40	45	
A*0301	2	16	17	19	26	28	29	37	43 45
A*0302	2	17	19	26	28	29	37	43	45
A*1101	2	9	17	19	26	28	29	31	37 43 45
A*1102	9	17	19	26	28	29	31	37	43 45
A*2301	2	4	13	16	19	25	32	37	40 45
A*2402	2	4	13	19	25	37	40	45	

A*2403	2	4	17	19	25	37	40	45							
A*2404	2	13	19	25	37	40	45								
A*2405	2	4	13	19	25	37	40	45							
A*2406	2	4	13	15	19	25	37	40	45						
A*2407	2	4	13	19	25	28	37	40	45						
A*2501	2	4	6	9	12	15	17	19	24	26	31	37	45		
A*2601	2	6	9	12	15	17	19	24	26	31	37	45			
A*2602	6	9	15	17	19	24	26	31	37	45					
A*2603	2	6	9	12	15	17	19	24	26	29	31	37	45		
A*2604	2	5	6	9	12	15	17	19	24	26	31	37	45		
A*2605	2	6	9	12	15	17	19	24	26	31	37	45			
A*2901	2	16	17	19	25	28	32	37	43	45					
A*2902	2	16	17	19	25	26	28	32	37	43	45				
A*3001	16	17	25	26	28	29	37	45							
A*3002	16	17	25	26	37	45									
A*3003	16	17	19	25	26	37	45								
A*3004	15	17	25	26	37	45									
A*3005	15	17	25	26	37	45									
A*3101	2	3	16	17	25	26	29	32	37	43	45				
A*3102	2	3	16	17	25	26	29	32	37	43	45				
A*3201	2	4	9	16	17	19	25	26	32	37	43	45			
A*3301	2	3	16	17	19	24	25	26	29	32	43	45			
A*3302	3	16	17	19	24	25	26	29	32	37	43	45			
A*3303	2	3	16	17	19	24	25	26	29	32	37	43	45		
A*3401	2	6	9	12	15	17	19	24	25	26	28	29	31	37	45

A*3402	2	16	17	19	24	25	26	28	29	31	37	45
A*3601	2	9	17	19	25	26	37	42	43	45		
A*4301	2	6	9	12	15	17	19	26	31	37	45	
A*6601	2	6	9	12	15	17	19	24	26	28	29	31 37 45
A*6602	2	6	9	12	15	17	19	24	26	28	29	31 37 45
A*68011	2	15	17	19	24	25	26	28	29	31	37	43 45
A*68012	2	15	17	19	24	25	26	28	29	37	43	45
A*6802	2	6	9	15	17	19	24	25	26	28	29	37 45
A*6901	2	6	16	17	19	24	25	26	28	29	31	37 45
A*7401	2	9	16	17	19	25	26	29	32	37	43	45
A*8001	2	13	18	26	37	43	45					

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HLA-B alleles

B*0702	2	9	14	17	19	24	30	31	35	37	40	42	44
B*0703	2	9	14	17	19	24	30	31	35	37	40	41	42 44
B*0704	2	9	14	17	19	24	30	31	35	37	40	44	
B*0705	2	9	14	17	19	22	24	30	31	35	37	40	42 44
B*0801	2	9	17	19	22	24	25	30	32	37	40	41	44
B*0802	2	9	17	19	22	24	25	32	37	40	41	44	
B*1301	2	6	10	11	17	18	19	22	32	33	37	45	
B*1302	2	11	17	18	19	22	32	33	37	45			
B*1303	2	5	11	17	18	19	22	32	33	37	45		
B*1401	2	9	17	18	23	24	25	26	30	31	40	41	45
B*1402	2	9	17	18	23	24	25	26	30	40	41	45	
B*1501	2	5	7	9	14	15	17	19	30	33	34	37	40 45
B*1502	2	5	6	9	10	14	17	18	19	24	26	30	33 34 37 45
B*1503	2	5	7	9	14	17	18	19	30	33	34	37	40 45
B*1504	2	5	9	14	15	17	19	26	30	33	34	37	45
B*1505	2	5	7	9	14	17	18	19	30	32	33	34	37 40 45
B*1506	2	5	7	8	9	14	17	18	19	30	33	34	37 40 45
B*1507	2	5	9	14	15	17	19	30	33	34	37	40	45
B*1508	2	5	7	9	14	15	17	19	24	30	34	37	40 45
B*1509	2	5	7	9	14	17	18	19	22	24	30	37	40 45

B*1510	2	5	7	9	14	17	18	19	24	30	37	40	45
B*1511	2	5	7	9	14	15	17	19	24	30	34	35	37
B*1512	2	5	7	9	13	14	15	19	30	33	34	37	40
B*1513	2	4	5	6	9	10	14	17	18	19	24	26	33
B*1514	2	5	7	9	14	15	19	30	33	34	37	40	45
B*1515	2	5	7	9	14	15	17	19	24	30	33	34	37
B*1516	2	4	5	6	11	14	17	18	19	34	37	40	45
B*1517	2	4	5	7	9	14	17	18	19	37	40	43	45
B*1518	2	5	7	9	14	17	18	19	24	30	34	37	40
B*1519	2	5	7	9	13	14	15	19	30	33	34	37	40
B*1520	2	5	6	10	11	17	18	19	20	30	32	33	34
B*1521	2	5	6	9	10	14	17	18	19	24	26	30	34
B*1522	2	5	7	9	14	15	17	19	24	30	34	37	40
B*1523	2	4	5	7	9	14	17	18	19	24	34	37	40
B*1524	2	4	5	7	9	14	15	17	19	33	34	37	40
B*1525	2	5	6	9	10	14	17	18	19	26	30	33	34
B*1526N	2	5	7	9	14	15	17	19	30	33	34	37	40
B*1528	2	5	7	9	14	15	17	19	30	33	34	37	40
B*1529	2	5	7	9	14	17	18	19	24	30	34	37	40
B*1801	2	7	9	17	18	19	24	25	30	32	33	34	40
B*1802	2	9	17	18	19	24	25	26	30	32	33	34	40
B*2702	2	4	9	14	17	18	19	26	32	37	40	43	45
B*2703	2	9	14	17	18	26	32	37	40	43	45		
B*27052	2	9	14	17	18	19	26	32	37	40	43	45	
B*27053	2	9	17	18	19	26	32	37	40	43	45		

B*2706	2	9	14	17	18	19	26	37	40	45
B*2707	2	9	14	17	18	19	22	32	37	40 44 45
B*2708	2	9	14	17	18	19	26	30	32	37 40 43 45
B*2709	2	9	14	17	18	19	26	32	37	40 45
B*3501	2	5	6	10	11	17	18	19	20	24 30 32 34 37 45
B*3502	2	5	6	10	11	17	18	19	22	24 30 32 37 45
B*3503	2	5	6	10	11	17	18	19	20	23 24 30 32 37 45
B*3504	2	5	6	10	11	17	18	19	20	22 24 30 32 37 45
B*3505	2	5	17	18	19	20	24	30	32	34 37 40 45
B*3506	2	5	6	10	11	17	18	19	20	22 23 24 30 32 37 45
B*3507	5	6	10	11	17	18	19	20	24	30 32 34 37 45
B*3508	2	5	6	10	11	17	19	20	24	30 32 34 37 42 45
B*3509	2	5	6	10	11	17	18	19	20	22 24 30 32 37 44 45
B*3510	2	5	6	10	11	17	18	19	20	30 32 34 37 45
B*3511	2	5	6	10	11	17	18	19	20	24 30 34 37 45
B*3512	2	5	9	10	17	18	19	22	24	30 32 37 45
B*3513	2	5	6	10	11	17	18	19	20	23 30 32 37 45
B*3701	2	9	17	19	23	25	32	33	37	45
B*3801	2	4	7	9	17	22	23	24	25	31 32 37 40 45
B*3802	2	7	9	17	22	23	24	25	31	32 37 40 45
B*39011	2	7	9	17	22	23	24	25	30	31 32 37 40 41 45
B*39013	2	7	9	17	22	23	24	25	30	31 32 37 40 41 45
B*39021	2	7	9	17	22	23	25	30	31	32 33 37 40 41 45
B*39022	2	7	9	17	19	22	23	25	30	31 32 33 37 40 41 45
B*3903	2	9	17	22	23	24	25	30	31	32 37 40 41 45

B*3904	2	7	9	17	22	23	24	25	30	32	37	40	41	45
B*3905	2	7	9	17	22	23	24	25	30	31	32	37	40	45
B*39061	2	9	17	22	23	24	25	30	31	32	37	41	45	
B*39062	2	9	17	22	23	24	25	26	30	31	32	37	41	45
B*3907	2	7	9	17	24	25	30	31	32	34	37	40	45	
B*3908	2	7	9	17	22	23	25	30	31	32	33	37	40	45
B*40011	2	7	9	14	17	18	19	22	30	32	33	37	40	44
B*40012	2	7	9	14	17	18	19	22	30	32	33	37	40	44
B*4002	2	9	14	17	18	19	22	30	32	33	37	40	44	45
B*4003	2	9	14	17	18	19	30	32	33	34	37	40	44	45
B*4004	2	6	10	11	14	17	18	19	22	30	32	33	37	44
B*4005	2	5	9	14	17	18	19	22	30	33	37	40	44	45
B*4006	2	9	14	17	18	19	22	26	30	32	33	37	44	45
B*4007	2	7	9	14	17	18	19	22	30	32	37	40	44	
B*4101	2	6	9	17	19	22	25	26	30	32	33	37	44	
B*4102	2	9	17	19	22	25	30	32	33	37	40	44		
B*4201	2	9	17	19	22	24	25	30	31	32	35	37	40	44
B*4402	2	5	9	10	19	32	33	37	43	45				
B*44031	2	5	9	10	18	19	32	33	37	43	45			
B*44032	2	5	6	9	10	18	19	26	32	33	37	43	45	
B*4404	2	9	10	19	25	32	33	37	42	43	45			
B*4405	2	5	9	10	19	32	33	37	45					
B*4406	2	4	5	9	10	19	24	32	37	43	45			
B*4501	2	5	6	11	19	20	30	32	33	37	45			
B*4601	2	5	7	9	14	15	17	19	30	34	37	40	45	

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B*4701	2	7	8	9	14	17	18	19	32	33	37	40	43	45
B*4801	2	9	14	17	18	19	22	30	31	32	33	37	40	44
B*4802	2	5	6	10	11	17	18	19	20	30	31	32	33	34 37 45
B*4901	2	4	5	6	11	17	18	19	20	33	37	45		
B*5001	2	5	6	11	17	18	19	20	30	33	37	45		
B*5101	2	4	5	9	17	18	19	22	24	26	45			
B*5102	2	4	5	9	17	18	19	22	24	26	37	45		
B*5103	2	4	5	9	18	19	22	24	26	45				
B*5104	2	4	5	6	9	10	17	18	19	22	24	26	45	
B*5105	2	4	5	9	17	19	22	24	26	32	37	42	45	
B*52011	2	4	5	9	17	18	19	22	26	33	45			
B*52012	2	4	5	9	17	18	19	22	26	33	45			
B*5301	2	4	5	6	10	11	17	18	19	20	24	32	34	37 45
B*5401	2	11	17	18	19	22	24	25	30	32	35	36	37	45
B*5501	2	11	17	18	19	22	24	25	30	35	37	45		
B*5502	2	11	17	18	19	22	24	25	30	32	35	37	45	
B*5601	2	5	11	17	18	19	22	24	30	32	35	37	45	
B*5602	2	5	7	17	18	19	22	24	30	32	35	37	40	45
B*5701	2	4	5	9	14	17	18	19	26	32	34	37	45	
B*5702	2	4	5	9	14	17	19	22	26	32	37	42	45	
B*5703	2	4	5	9	14	17	18	19	22	26	32	37	45	
B*5801	2	4	5	6	10	11	17	18	19	20	32	34	37	45
B*5802	2	4	5	11	17	18	19	20	32	34	37	40	45	
B*5803	2	4	5	11	17	18	19	20	32	34	37	45		
B*5901	2	4	11	17	18	19	22	24	25	32	37	45		

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B*67011	2	7	9	17	22	23	24	25	30	31	32	35	37	40	45
B*67012	2	7	9	17	19	22	23	24	25	30	31	32	35	37	40
B*7301	14	17	18	19	23	24	30	32	44	45					
B*7801	2	5	9	17	18	19	22	24	26	30	41	45			
B*7802	2	5	9	17	18	19	22	24	26	30	45				
B*8101	2	9	14	17	18	19	22	24	30	31	32	35	37	40	44

HLA-C Alleles

Cw*0101	17	19	20	25	30	36	37	40	42	44					
Cw*0102	17	19	20	25	30	36	37	40	42	44	45				
Cw*0201	7	15	17	19	20	34	37	40	44						
Cw*02021	7	15	17	19	20	34	36	37	40	44	45				
Cw*02022	7	15	17	19	20	34	36	37	40	44	45				
Cw*0302	2	5	6	14	17	18	19	20	26	30	34	36	45		
Cw*0303	2	5	6	10	14	17	18	19	20	26	30	36	45		
Cw*0304	2	5	6	10	14	17	18	19	20	26	30	36	45		
Cw*0401	2	7	8	14	17	19	23	25	37	40	42	45			
Cw*0402	2	7	8	14	17	19	23	25	37	40	45				
Cw*0501	6	7	11	19	20	21	23	25	36	37	40	42	44		
Cw*0602	11	14	15	17	19	20	25	34	37	40	44	45			
Cw*0701	6	7	11	17	18	19	20	25	30	34	36	37	40	44	45
Cw*0702	7	17	18	19	20	25	30	34	36	37	40	44	45		
Cw*0703	7	17	18	19	20	30	34	36	37	40	44	45			

CW*0704	6	11	17	19	20	23	25	30	37	44					
CW*0801	6	7	11	14	17	18	19	20	23	25	30	36	37	40	44
CW*0802	6	7	11	17	19	20	23	25	30	36	37	40	42	44	
CW*0803	6	7	11	14	17	18	19	20	23	25	30	36	40	44	
CW*1201	7	14	15	17	19	20	25	30	37	40	44	45			
CW*12021	7	14	15	17	19	20	25	30	34	36	37	40	44	45	
CW*12022	7	14	15	17	19	20	25	30	34	36	37	40	44	45	
CW*1203	11	14	15	17	19	20	25	30	34	36	37	40	44	45	
CW*1301	7	14	15	17	19	20	25	30	34	37	40	44	45		
CW*1401	2	14	17	19	20	25	30	34	36	37	40	42			
CW*1402	2	14	17	19	20	25	30	34	36	37	40	42	45		
CW*1403	2	14	17	19	20	25	30	34	36	37	40	42	45		
CW*1501	6	10	11	14	17	18	19	20	25	36	37	45			
CW*1502	6	10	11	14	17	18	19	20	25	36	37	44	45		
CW*1503	6	10	11	14	17	18	19	20	25	36	37	44	45		
CW*1504	6	10	11	14	17	18	19	20	25	34	36	37	44	45	
CW*1505	6	10	11	14	17	18	19	20	23	25	36	37	44	45	
CW*1601	11	17	19	20	25	30	34	36	37	40	45				
CW*1602	11	17	19	20	25	34	36	37	40	45					
CW*1603	11	14	17	19	20	25	30	34	36	37	40	44	45		
CW*1701	6	11	14	17	18	19	20	23	36	37	44	45			

Table 3

The location of the target sequences for URSTO probes
in HLA class I genes

Sequence range	No. of probes	Probe identity
1. 92-109	1	31
2. 113-130	1	2
3. 212-229	1	36
4. 236-299	7	24,28,19,35,41,3,33 (a)
5. 293-310	1	29
6. 307-324	2	4,30
7. 333-430	14	6,8,10,26,11,20,7,40,9 (b) 23,12,34,43,22
8. 454-471	1	44
9. 478-495	1	14
10. 520-578	9	5,25,32,15,16,13,17,18,42 (c)
11. 580-597	1	37
12. 597-614	1	45

The probe identity numbers are same as in Table 1; a, b and c indicate the hypervariable sequence regions which are present in the three loci. The sequence range refers to the base positions in exons 2 and 3.

The panel of probes used in the invention generally consists of a sufficient number of probes to uniquely identify the majority of the alleles of the polyallelic gene. For example, the panel of probes may be selected so as to uniquely identify at least 50%, at least 75%, at least 90%, at least 95%, at least 99% or 100% of the alleles in the polyallelic system. The exact number of probes will vary depending on the gene, but is typically from 10 to 100, preferably from 20 to 70 or from 30 to 50. Each probe may recognise a sequence motif that is present in, for example, from 2 to 30, from 2 to 20, for 4 to 20 or from 6 to 16 alleles of the polyallelic gene.

When the invention is applied to the HLA class I system, the panel of probes preferably comprises from 20 to 40 probes which each recognises the motif recognised by one of the probes set out in Table 1. Each of the probes in the panel may have a sequence at least 40%, at least 60%, at least 80%, at least 90% or at least 95% identical to either (i) a sequence of one of the probes set out in Table 1 or (ii) a sequence complementary to a sequence of one of the probes set out in Table 1. A probe in the panel may have a sequence that is shifted along the HLA class I gene sequence by a certain number of nucleotides compared to a probe set out in Table 1; for example, a probe may be shifted along by from 1 to 10 nucleotides (e.g. from 1 to 5 nucleotides) in either a 5' or a 3' direction compared to a probe set out in Table 1.

The probes used in the invention may be labelled with any one of a variety of detectable labels in order to facilitate their detection. Examples of suitable labels include digoxigenin (which may be detected using an anti-digoxigenin antibody coupled to alkaline phosphatase), radiolabels, biotin (which may be detected by avidin or streptavidin conjugated to peroxidase) and fluorescent labels (e.g. fluorescein and rhodamine).

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The kit according to the invention may comprise a database which indicates which probes in the panel in the kit recognise each allele of the polyallelic gene. The database may be a paper database or a computer database.

- 5 The database may be compiled by examining the sequences of the allele of the polyallelic genes and noting the probes which would be expected to bind to each allele. The accuracy of a database compiled by such a technique may be verified by experimentally determining which alleles are
10 bound by each probe in the panel. Table 2 contains a database showing which of the 40 probes in Table 1 bind specific HLA class I alleles. The kit may also contain one or more known alleles as control(s). Such controls can be used to verify that an experiment carried out using the kit
15 has worked correctly.

- It is highly desirable that the sample of allele used in the invention comprises one allele only and is not contaminated by other alleles of the same gene. The
20 presence of two similar alleles in the sample can give confusing results and prevent conclusive identification of the alleles. Individuals are often heterozygous with respect to the alleles of a particular gene, i.e. individuals often have two different alleles of the same
25 gene, and these alleles normally need to be separated before carrying out the invention.

- In view of the fact that the difference between two alleles of a gene can be as little as one nucleotide, it is often
30 difficult to separate the alleles from a mixture of the alleles. These difficulties are increased in genes which have a very large number of different alleles, such as the major histocompatibility complex (MHC) genes (e.g. the human leucocyte antigen (HLA) class I genes which have 222
35 known alleles).

A new method for separating alleles of a gene from a

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mixture of alleles has now been found, which is referred to herein as "Complementary Strands Analysis" (CSA). This method is described in detail in an international (PCT) patent application in the name of the Anthony Nolan Bone Marrow Trust being filed on the same day as this application. The method comprises

- (i) amplifying the alleles in the mixture of alleles;
- (ii) hybridising single strands of the amplified alleles with a complementary strand of a reference allele to form duplexes; and
- (iii) separating the duplexes.

The different alleles in the original mixture give rise to duplexes having different numbers of mismatches compared to a selected complementary reference DNA strand. This allows the duplexes to be separated by, for example, gel electrophoresis. The separated duplexes can then be analysed by the method of the invention to identify the alleles that were present in the original mixture.

A preferred form of the CSA method comprises

- (i) amplifying the mixture of alleles employing a pair of primers in which one of the primers carries a ligand, so as to produce an amplified mixture of double-stranded alleles in which one of the strands carries a ligand;
- (ii) contacting the amplified mixture of double-stranded alleles with a receptor on a solid support under conditions such that the ligand binds to the receptor;
- (iii) separating the mixture of double-stranded alleles into single-strands and removing the strands that are not bound to the support by the ligand;

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(iv) recovering the remaining strands from the support;

5 (v) mixing the recovered strands with a complementary strand of a reference allele so as to form duplexes; and

(vi) separating the duplexes.

10 Another form of the CSA method comprises

(i) amplifying the alleles in the mixture employing a pair of primers in which one of the primers carries a high molecular weight molecule, so as
15 to produce an amplified mixture of double-stranded alleles in which one of the strands carries a high molecular weight molecule;

(ii) separating the mixture of double-stranded alleles
20 into single strands;

(iii) mixing the single strands with a complementary strand of a reference allele so as to form duplexes; and

25

(iv) separating the duplexes.

This form of CSA overcomes the need for solid support systems by conjugating one primer of a pair of primers
30 directly to a high molecular weight molecule (e.g. a protein). The amplified product after hybridisation can be applied directly to a separating gel. The high molecular weight conjugates are retained in the gel compared to the duplex without attachment of the high molecular weight
35 molecule.

In yet another form of the CSA method, there is provided a

method for separating an allele from a mixture of alleles, which method comprises

- 5 (i) amplifying a single strand of each of the alleles in the mixture;
- (ii) mixing the amplified single strands with a complementary strand of a reference allele so as to form duplexes; and
- 10 (iii) separating the duplexes.

The amplification of the single strand can be done, for example, by asymmetric PCR.

15 This form of CSA overcomes the need for both solid support systems and conjugation of one primer of a pair to a high molecular weight molecule. However, in the embodiment it is possible to use a primer carrying a ligand such as a
20 hapten in order to facilitate capture of the amplified strand with a receptor such as an antibody and separation of the amplified strand from other components in the amplification mixture.

25 In each of the above forms of CSA, the reference allele may be provided in single-stranded form by essentially the same steps as used to provide the test alleles in single-stranded form.

30 The CSA method provides an improvement over prior methods for separating alleles. The advantages offered by CSA can be summarised as follows:

- 35 (a) The method provides a high resolution between different alleles and differences of as little as one nucleotide between alleles can be detected.

- 36 -

- (b) The method allows simultaneous and rapid processing of a large number of samples.
- (c) The method is comparatively inexpensive to perform, particularly when compared to prior methods which achieve a high level of resolution.
- (d) The method uses techniques that can be performed easily without recourse to complex and expensive technology.

The reference allele used in the CSA method generally has a known sequence. Further, the reference allele is usually chosen so as to have a similar allotype to an allotype that at least one of the test alleles is suspected of having. For example, it may be known that a test allele is of the HLA-A02 type from serological data, but it may not be known which of the seventeen A02 sub-types the allele is. In this case, the reference allele may be chosen to be of sub-type A0201 and the method of the present invention could then be used to determine which of the A02 sub-types the test allele is.

The reference strand may be obtained from (a) a homozygous source, (b) a heterozygous source from which individual strands are isolated by gel separation after amplification steps or (c) DNA synthesis. There are now about 500 internationally recognised cell lines which contain HLA alleles of known sub-type and these cell lines can be used as a source of reference alleles.

In the CSA method, the amplification steps may be carried out by polymerase chain reaction (PCR).

The ligand/receptor system used in the CSA method may, for example, be the biotin/streptavidin system. Direct conjugation of the primer via a linking group, such as

short poly A, to the beads is an alternative. When the biotin/streptavidin system is used, one of the primers used in each of the amplification steps may be labelled with biotin, so that when the amplification reaction is carried out double-stranded DNA is produced in which one strand carries a biotin label. The double-stranded DNA may then be bound to a solid support coated with streptavidin.

The solid support used in the CSA method is typically magnetic beads. However, other supports may be used, such as the matrix of an affinity chromatography column. When the support is in the form of magnetic beads, the two strands of the amplified DNA are separated by attracting the beads to a magnet and washing the beads under conditions such that the double-stranded DNA dissociates into single-strands. The dissociation is typically performed by incubating the beads (e.g. three times) under alkaline conditions (e.g. 0.1 M or 0.15 M NaOH) at room temperature for about 5 or 10 minutes. Usually, the strand which is not bound to the support by the ligand is then discarded, although it is equally possible to retain the strand that is not bound to the support and discard the strand that is bound to the support.

The strand that remains attached to the support may be recovered from the support by incubating the support under conditions such that the ligand/receptor complex dissociates. When the biotin/streptavidin system is used, the support is typically heated to e.g. 95°C for about 5 minutes; this ensures denaturation of the streptavidin molecule to release the biotinylated single strand which is then recovered.

At this stage, there have been provided a single-stranded unknown allele and the complementary strand of a reference allele. The two strands are then mixed together under conditions in which they hybridise to form duplexes.

Typically, the hybridisation step is performed by heating the mixture of strands at about 95°C for about 3 min, at about 70°C for about 5 min and then at about 65°C for about 45 min.

5

Under these conditions, duplexes are formed which can subsequently be separated by gel electrophoresis (e.g. polyacrylamide gel electrophoresis). The electrophoresis may be carried out under denaturing or non-denaturing conditions. The use of denaturing conditions may enhance separation.

10

As an alternative separation technique to gel electrophoresis, high pressure liquid chromatography (HPLC) may be used.

15

In the embodiment of the CSA method in which one of the pair or primers is conjugated to a high molecular weight molecule, the molecule may be a protein such as bovine serum albumin (BSA). The molecular weight of the high molecular weight molecule is such that it causes the DNA molecule to which it is attached to be sufficiently retarded in the separation step (e.g. the electrophoresis step) to allow the DNA molecule to be separated from a duplex without a high molecular weight compound attached. For example, the molecular weight of the high molecular weight molecule may be from 10 to 200 kDa, preferably 20 to 100 kDa.

20

25

The invention may be used to match a prospective donor in a tissue or organ transplant operation with a prospective recipient. In particular, the invention may be used to identify the alleles of the prospective recipient and donor, and hence to determine whether they have compatible alleles. The prospective recipient and donor may, for example, be a prospective recipient and donor in a bone marrow or kidney transplant operation.

30

35

Other proposed uses of the invention include determination of the paternity of an individual by identifying one (or more) of his alleles to see if it is the same as a corresponding allele of a putative father. The invention
5 may also be used in forensic medicine to determine the origin of a sample of body tissue or fluid, as a follow up technique in treatment of haematological malignancies or inherited disorders, in adoptive immunotherapy, and in identification of bacteria and viruses.

10

The following example illustrates the invention.

EXAMPLE

15 METHODS

1.- Locus specific amplification of HLA class I genes

For typing purposes, amplification of exons 2 and 3 is
20 desirable, and the primers were therefore selected to amplify the stretch of the genome between intron 1 and intron 3. The localisation and nucleotide sequences of the HLA locus-specific primers used are given in the reagents section.

25

PCR reactions were performed in a total volume of 100 μ l using 1 μ g of genomic DNA and 25 pmoles of each locus-specific primer. The 3' primer was biotinylated at the 5' end. This arrangement ensures the incorporation of the
30 biotinylated primer onto the amplified antisense DNA strand. PCR conditions are given in the following table.

- 40 -

Thermocycling conditions**A, B and Cw loci**

	95° C	4 min.	1 cycle
5	95° C	30 sec.	
	65° C	50 sec.	33 cycles
	72° C	30 sec.	
	72° C	8 min.	1 cycle

10 2.- Separation of the amplified DNA strands**a) Removal of non-biotinylated strand:**

Magnetic beads with covalently coupled streptavidin on the surface were added to the PCR product and incubated for 30 minutes at 43° C. In this way the amplified PCR product was immobilised by the interaction of biotin and streptavidin. After incubation, the tubes were placed against a magnet and the beads were washed with washing buffer to remove the remaining PCR reaction components.

20

The non-biotinylated DNA strand was then dissociated from the beads by incubation with 0.1 M NaOH at room temperature (r.t.) for 10 minutes. Following this the beads were washed to remove excess NaOH and resuspended in 50 µl of hybridisation buffer.

25

b) Removal of biotinylated DNA strand:

The bead suspension was heated at 95°C for 5 minutes, which ensures denaturation of the streptavidin molecule to release the biotinylated amplified anti-sense single strand which was then removed and placed in a clean tube.

30

At this stage, the isolates contained single biotinylated DNA strands from each allele.

35

3.- Hybridisation with locus specific reference sense single strand DNA

The biotinylated anti-sense strand(s) from above were mixed
5 with a locus specific reference sense strand, Rf A, Rf B
and Rf C for HLA-A, B and C respectively (see below), and
the mixture was heated at 95°C for 3 min., incubated at
70°C for 5 min. and then at 65°C for 45 min. Under these
conditions the sense and anti-sense strands were
10 hybridised. The heteroduplexes formed by each allele
antisense strand with the locus specific reference sense
strand could subsequently be separated from each other by
electrophoresis in non-denaturing polyacrylamide gel.

15

4.- Preparation of locus specific reference sense single strand DNA.

DNA was extracted from three homozygous 10th IHW cell
20 lines. The following cell lines were selected as locus
specific reference DNA: STEINLIN (HLA-A*0101), SP0010 (HLA-
B*4402) and STIENLIN (HLA-Cw*0701).

The PCR conditions for amplification were as above, with
25 the exception that in each case the locus-specific 5'
primer was biotinylated (5' end). The PCR products were
analysed by PAGE to assess the fidelity of the
amplification and in all cases a single band was obtained.

30 The biotinylated single sense strand was isolated as
described above and its purity was tested by a
heating/annealing cycle of the sample followed by agarose
electrophoresis. In each case only a single band of the
expected size was observed.

35

5.- Separation of alleles

This step is critical for the separation of the allelic products from heterozygous subjects and from coamplified
5 non-specific products.

The heteroduplexes formed as described in step 3 were separated from each other by electrophoresis analysis which was performed on an 8% non-denaturing polyacrylamide gel at
10 room temperature (200 volts for 6 hours). The DNA was visualised by ethidium bromide staining and U.V. light.

The heteroduplexes from heterozygote individuals were resolved into two bands, while DNA from homozygote subjects
15 produced a single band.

6.- Identification of HLA class I alleles

The bands were excised from the gel from which the DNA was eluted and blotted on the same membrane for three loci.
20 For heterozygous subjects two dots per locus were prepared. Several subject samples were blotted on each membrane. According to the number of the URSTO probes several membranes were prepared.

25 The oligonucleotide probes were labelled with digoxigenin (DIG) at the 3' end (Boehringer, according to specification).

30 Hybridisation and washing solutions contained TMACl (3M), and membranes were hybridised at 54°C and washed at 58°C. Oligonucleotide binding was detected by chemiluminescence; anti-DIG-antibody conjugated to alkaline phosphatase and CSPD were added to membranes. After incubation the
35 chemiluminescence was detected by X-ray films.

RESULTS

A.- Separation of alleles by CSA

5 Anti-sense strands from more than 20 samples were isolated,
hybridised with the HLA-A locus specific reference sense
strand (STEINLIN A*0101), and analysed by 8% non-denaturing
polyacrylamide gel electrophoresis. In all cases there was
a good correlation between the number of bands observed and
10 the zygosity of the sample. For example, two bands were
seen for heterozygous samples whereas one band was seen for
homozygous samples. These bands were always observed in
the same area of the gel, the lower half nearest to the
anode. A representative autoradiograph from CSA analysis
15 is presented in Figure 2B.

B.- Identification of alleles by URSTO

For initial testing of the labelled URSTO probes, DNA from
20 10 homozygous EBV transformed B cell lines set out in the
following table was amplified for the HLA-A, B and C loci
using locus specific primers and blotted onto 10 nylon
membranes.

Specificity of the 10 homozygous cell lines used in URSTO

	STEINLIN	A*0101	B8	C*0701
5	LBUF	A*3001	B*1302	C*0601
	BM14	A3	B7	C7
	JBUSH	A32	B38	C*1203
10	BTB	A2	B27	C1
	WT47	A32	B44	C5
15	SWEIG007	A*2902	B*4002	C*02022
	BM92	A*2501	B*5101	C1
	SPL	A31	B62	C1
20	SPOO10	A2	B*4402	C5

Four URSTO probes (P3, P4, P5 and P29) labelled with DIG were then hybridised to these membranes under specific conditions: 54°C for 90 minutes in TMAcI solution. The membranes were then washed (X3) under stringent conditions: 58°C for 10 min. in TMAcI wash solution. Detection was performed by anti-DIG alkaline-phosphatase conjugate, followed by development with CSPD.

It was found that the four URSTO probes gave the expected pattern (see the following table).

Results of 4 URSTO probes

Allelic specificity		URSTO probes			
		<u>P3</u>	<u>P4</u>	<u>P5</u>	<u>P29</u>
5	A*0101	-	-	-	-
	B8	-	-	-	-
	C*0701	-	-	-	-
10	A*3001	-	-	-	+
	B*1302	-	-	-	-
	C*0601	-	-	-	-
	A3	-	-	-	+
	B7	-	-	-	-
15	C7	-	-	-	-
	A32	-	+	-	-
	B38	-	+	-	-
	C*1203	-	-	-	-
	A2	-	-	-	+
20	B27	-	-	-	-
	C1	-	-	-	-
	A32	-	+	-	-
	B44	-	-	+	-
	C5	-	-	-	-
25	A*2902	-	-	-	-
	B*4002	-	-	-	-
	C*02022	-	-	-	-
	A*2501	-	+	-	-
	B5101	-	+	+	-
30	C1	-	-	-	-
	A31	+	-	-	+
	B62	-	-	+	-
	C1	-	-	-	-
	A2	-	-	-	+
35	B*4402	-	-	+	-
	C5	-	-	-	-

Tests with all 40 probes and a large number of internationally defined samples (International Histocompatibility workshop cell lines) indicate that each allele tested gave the pattern shown in Table 2 (see

5 Figures 3 and 4).

A list of HLA class I alleles which have been isolated and identified by URSTO is set out in the following table:

5 HLA class I alleles which have been isolated
and identified by URSTO method

HLA-A (n=33)

10 A*0101, A*0102, A*0201, A*0202, A*0203, A*0204, A*0205,
A*0206, A*0207, A*0208, A*0209, A*0210, A*0211, A*0212,
A*0213, A*0216, A*0217, A*0301, A*1101, A*2301, A*2402,
A*2403, A*2501, A*2601, A*2902, A*3001, A*3002, A*3101,
A*3201, A*3301, A*6601, A*6602, A*6802

15 HLA-B (n=30)

B*0702, B*0801, B*1302, B*1402, B*1501, B*1502, B*1520,
B*1801, B*3501, B*3701, B*3801, B*4001, B*4002, B*4101,
B*4201, B*4402, B*4403, B*4601, B*4701, B*4801, B*4901,
B*5001, B*5101, B*5201, B*5301, B*5502, B*5701, B*5801,
20 B*5802, B*6701

HLA-Cw (n=18)

Cw*0102, Cw*0202, Cw*0302, Cw*0303, Cw*0304, Cw*0401,
Cw*0501, Cw*0602, Cw*0701, Cw*0702, Cw*0704, Cw*0802,
25 Cw*1202, Cw*1203, Cw*1402, Cw*1502, Cw*1601, Cw*1701

Number of different heterozygous combinations tested: HLA-A
19, HLA-B 14, and HLA-Cw 11.

30

In the identification of the alleles set out in the above
table, DNA was extracted from 63 B-lymphoblastoid cell
lines; these included 22 heterozygous and 41 homozygous
lines. After PCR amplification with locus specific primers
35 as described above the anti-sense single strands were
isolated and hybridised as described above with the
appropriate reference strands (A*0101, B*4402, Cw*0701).

- 48 -

The allelic bands were resolved in non-denaturing PAGE and eluted from low melting point agarose as described above. The DNA from the isolated bands was blotted on 40 nylon membranes (as in Figure 3) and hybridised with URSTO probes. Alleles were identified by comparison of patterns with those in Table 2.

REAGENTS

10 A) Nucleotide sequences of primers used for locus-specific amplification:

5' A locus primer: GAA ACG/C GCC TCT GT/CG GGG AGA
AGC AA

15 (Intron 1: 21-46)

3' A locus primer: TGT TGG TCC CAA TTG TCT CCC CTC
(Intron 3: 66-89)

20 5' B locus primer: GGG AGG AGC GAG GGG ACC G/CCA G
(Intron 1: 36-57)

3' B locus primer: GGA GGC CAT CCC CGG CGA CCT AT
(Intron 3: 37-59)

25 5' C locus primer: AGC GAG GG/TG CCC GCC CGG CGA
(Intron 1: 42- 61)

3' C locus primer: GGA GAT GGG GAA GGC TCC CCA CT
30 (Intron 3: 12-35)

B) Buffers:

35 Washing buffer: 10 mM Tris-HCl pH 7.5
1.0 mM EDTA
2.0 M NaCl

- 49 -

5	Hybridisation buffer:	20 mM	Tris-HCl pH 8.4
		50 mM	KCl
	PCR buffer:	20 mM	Tris-HCl pH 8.4
10		50 mM	KCl
		0.2 mM	MgCl ₂
	TE buffer	10 mM	Tris-HCl pH 7.5
		1 mM	EDTA

C) Various

- 15 Dynabeads M-280 Streptavidin (10 mg/ml)
- Magnetic particle concentrator -Dyna MPC
- Nylon membranes, positively charged (Boehringer Mannheim)
- 20 CSPD-Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chole)tricyclo[3.3.1.1.3,7] decan}-4-yl) Boehringer Mannheim
- 25 DIG Oligonucleotide 3' -End Labeling Kit (Boehringer Mannheim)
- Anti-digoxigenin-AP Fab fragments (Boehringer Mannheim)
- 30 A Thermal cycler (PTC-200 Peltier Thermal Cycler MJ Research)
- Ultrapure dNTP set, 2' -Deoxynucleoside 5' -Triphosphate (Pharmacia Biotech)
- 35 Taq DNA Polymerase (Gibco BRL)
- 50 mM MgCl₂

0.1 M NaOH

SeaPlaque Agarose (Flowgen Instruments Ltd)

- 5 Protogel, 30% Acrylamide and 0.8% Bisacrylamide (National Diagnostics)

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CLAIMS

1. A method for identifying an unknown allele of a polyallelic gene, which method comprises

- 5 (i) contacting the unknown allele with a panel of probes, each of which recognises a sequence motif that is present in some alleles of the polyallelic gene but not in others;
- 10 (ii) observing which probes recognise the unknown allele so as to obtain a fingerprint of the unknown allele; and
- 15 (iii) comparing the fingerprint with fingerprints of known alleles.

2. A method according to claim 1 wherein the polyallelic gene is a human leucocyte antigen (HLA) gene.

20 3. A method according to claim 2 wherein the HLA gene is an HLA class I gene or an HLA class II gene.

25 4. A method according to claim 1, 2 or 3 wherein the panel of probes consists of from 20 to 70 probes.

30 5. A method according to claim 3 wherein the HLA gene is an HLA class I gene and the panel of probes comprises from 20 to 40 probes which each recognises the motif recognised by one of the probes set out in Table 1.

6. A method according to claim 5 wherein each of the probes has a sequence at least 40% identical to either (i) a sequence of one of the probes set out in Table 1 or (ii) a sequence complementary to a sequence of one of the probes set out in Table 1.

7. A method according to claim 5 wherein the panel

of probes comprises the 40 probes set out in Table 1.

8. A method according to any one of the preceding claims wherein the unknown allele has been separated from a mixture of alleles of the polyallelic gene by

- (i) amplifying the alleles in the mixture of alleles;
- (ii) hybridising single strands of the amplified alleles with a complementary strand of a reference allele to form duplexes; and
- (iii) separating the duplexes.

9. A method according to claim 8 comprising

- (i) amplifying the mixture of alleles employing a pair of primers in which one of the primers carries a ligand, so as to produce an amplified mixture of double-stranded alleles in which one of the strands carries a ligand;
- (ii) contacting the amplified mixture of double-stranded alleles with a receptor on a solid support under conditions such that the ligand binds to the receptor;
- (iii) separating the mixture of double-stranded alleles into single-strands and removing the strands that are not bound to the support by the ligand;
- (iv) recovering the remaining strands from the support;
- (v) mixing the recovered strands with a complementary strand of a reference allele so as to form duplexes; and
- (vi) separating the duplexes.

10. A method according to claim 9, modified by recovering the strands that do not bind to the support instead of those that bind to the support, and mixing these recovered strands with the reference allele strand in step
5 (v).

11. A method according to any one of the preceding claims wherein the unknown allele is from a prospective donor or a prospective recipient in a tissue or organ
10 transplant operation.

12. A method according to any one of the preceding claims wherein each probe in the panel recognises a sequence motif that is present in from 2 to 20 alleles of
15 the polyallelic gene.

13. A kit for identifying an unknown allele of a polyallelic gene, which kit comprises a panel of probes, each of which probes recognises a sequence motif that is
20 present in some alleles of the polyallelic gene but not in others.

14. A kit according to claim 13 also comprising a database which indicates which probes in the panel
25 recognise each allele of the polyallelic gene.

15. A kit according to claim 13 or 14 also comprising a known allele as a control.

30 16. A kit according to claim 13, 14 or 15 wherein the panel of probes is selected so as to recognise motifs of an HLA gene.

17. A kit according to claim 16 wherein the HLA gene
35 is an HLA class I gene or an HLA class II gene.

18. A kit according to any one of claims 13 to 17

wherein the panel consists of from 20 to 70 probes.

19. A kit according to claim 17 wherein the HLA gene is an HLA class I gene and the panel of probes comprises
5 from 20 to 40 probes which each recognises the motif recognised by one of the probes set out in Table 1.

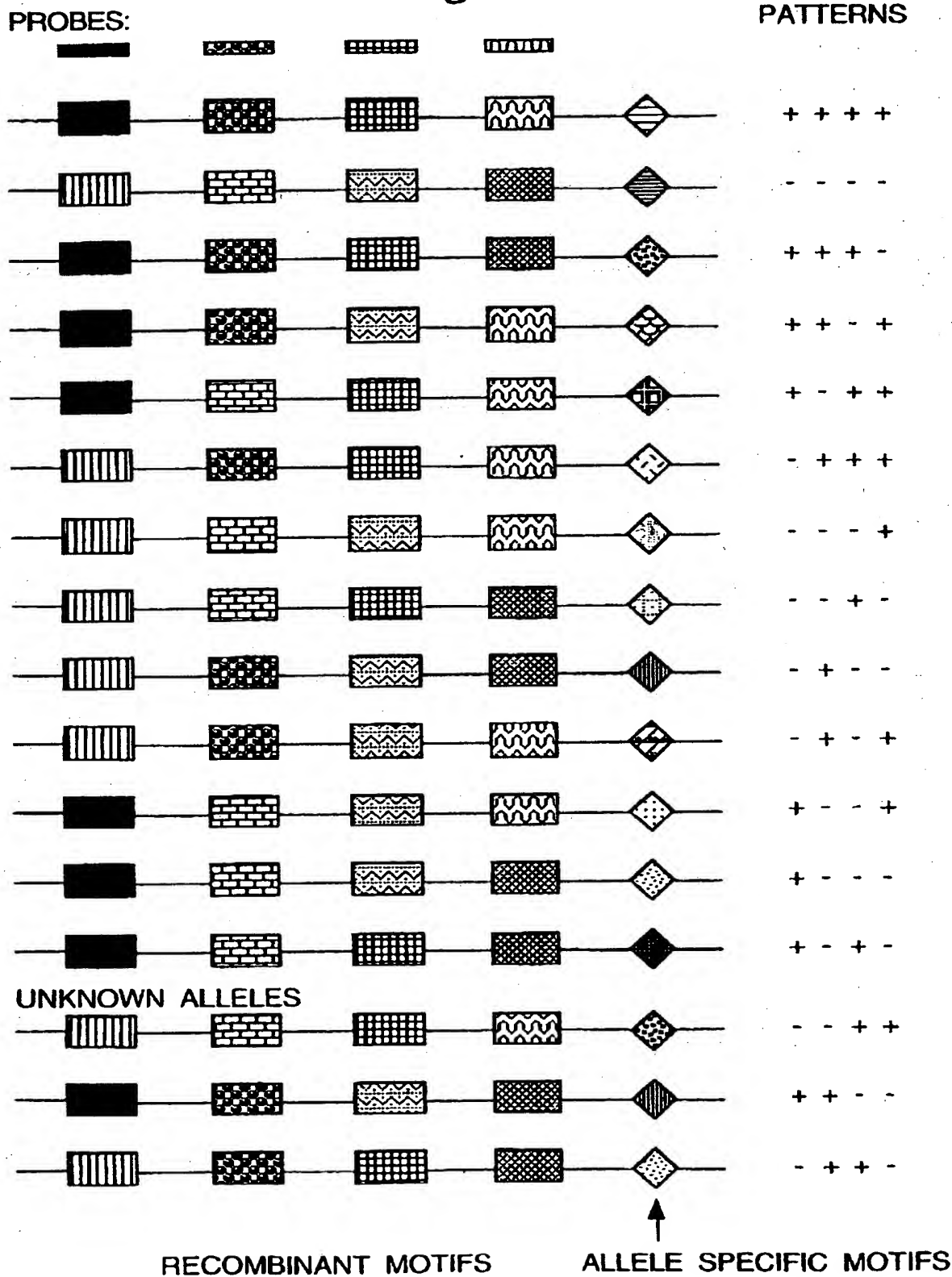
20. A kit according to claim 19 wherein each of the probes has a sequence at least 40% identical to either (i)
10 a sequence of one of the probes set out in Table 1 or (ii) a sequence complementary to a sequence of one of the probes set out in Table 1.

21. A kit according to claim 19 wherein the panel of
15 probes comprises the 40 probes set out in Table 1.

22. A kit according to any one of claims 13 to 21 wherein each probe in the panel recognises a sequence motif that is present in from 2 to 20 alleles of the polyallelic
20 gene.

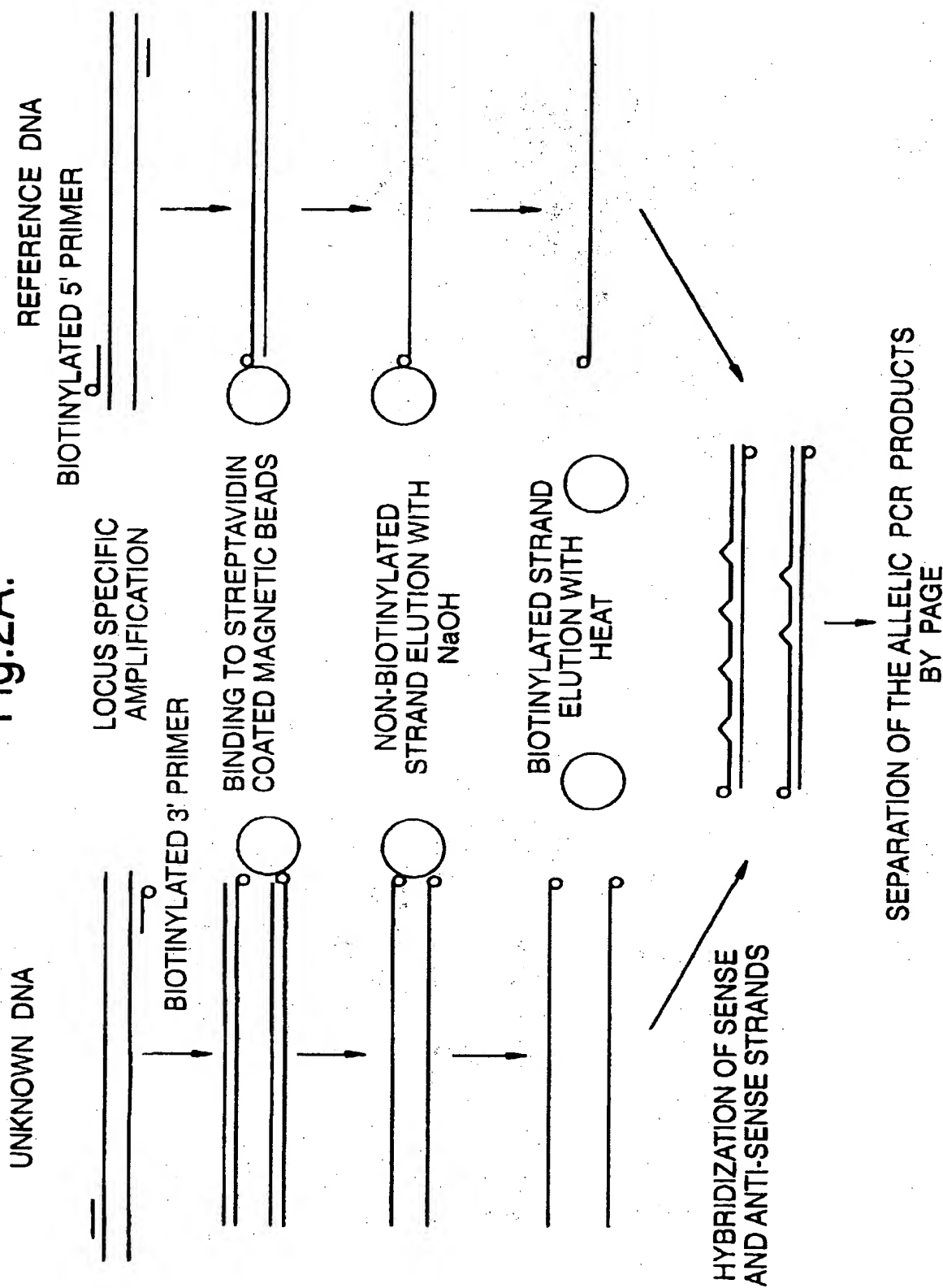
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Fig.1.



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Fig.2A.



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Fig.2B.

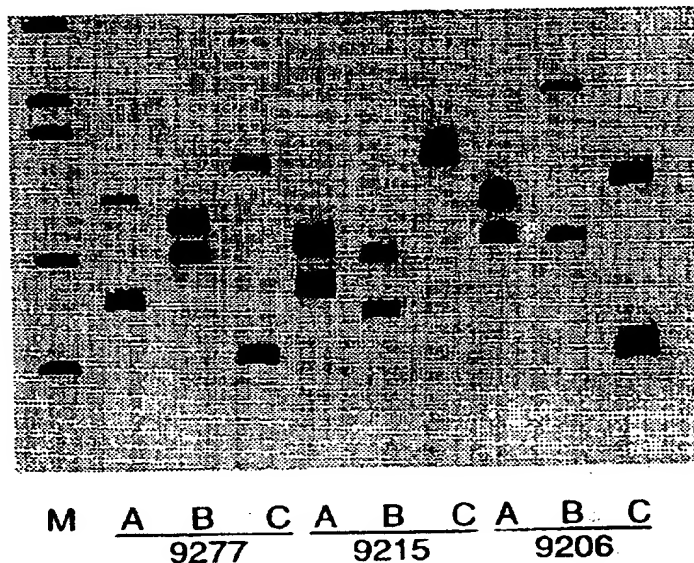


Fig.3.

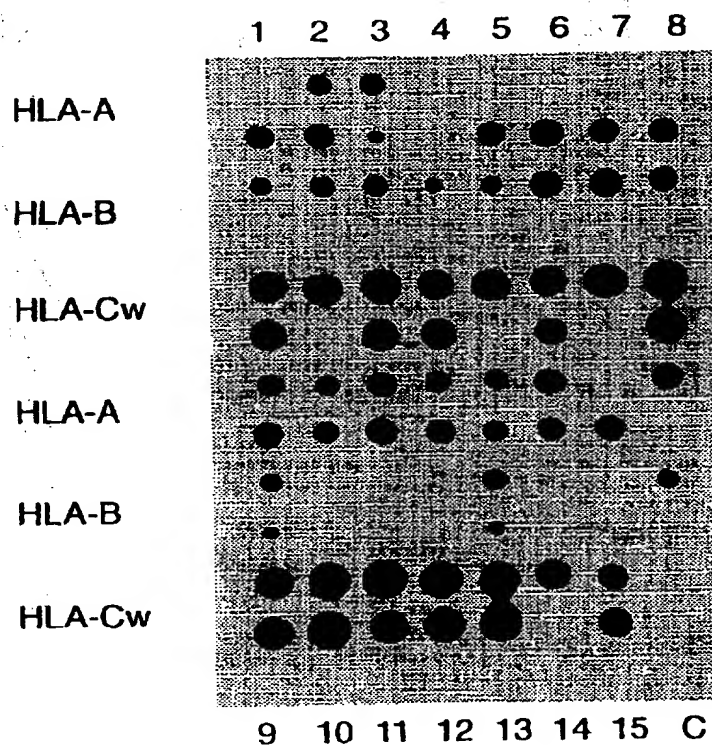


Fig.4.

